Structure and Function of Ovotransferrin

I. PRODUCTION OF IRON-BINDING FRAGMENTS FROM IRON-OVOTRANSFERRIN

BY THE ACTION OF IMMOBILIZED SUBTILISIN. PURIFICATION AND
CHARACTERIZATION OF THE FRAGMENTS*

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Immobile subtilisin Novo was used for the cleavage of iron-saturated ovotransferrin (Fe₂OT) into separate NH₂- and CO₂H-terminal iron-binding fragments, designated as FeNF and FeCF, respectively. The M₀ of each fragment is 39,000. The purified fragments show major differences in the content of histidine, alanine, and methionine. Both apo-NH₂- and apo-CO₂H-terminal fragments are able to bind one ferric ion per molecule. FeCF is more resistant than FeNF to dissociation at acid pH and to subtilisin action.

FeNF and FeCF are immunochemically distinct. However, equal mixtures of the two show immunochemical reaction indistinguishable from intact Fe₂OT. The iron-binding sites of FeNF and FeCF are very similar to each other on the basis of visible absorption and CD spectra. The major difference in the backbone conformations between FeNF and FeCF is in the a-helical content of FeCF which is twice that of FeNF. Individually, fragments show quantitative differences in the electron paramagnetic resonance spectra; however, equal mixture of the two fragments produce EPR spectra very similar to that of the intact Fe₂OT.

These studies indicate that subtilitic cleavage of Fe₂OT does not produce significant change in the iron-binding capacity or the conformation of the separated iron-binding domains.

Transferrins are a group of homologous iron-binding glycoproteins (Mₒ ≈ 80,000) which are widely distributed in various biological fluids (1, 2); they possess two binding sites for ferric iron and are involved in the transport of iron to the developing red cells for the synthesis of hemoglobin.

It has been suggested that the two sites may have different functions in iron transport in vivo (3, 4). However, in the homologous red cell transferrin systems of human, rabbit, and chicken, in vivo studies indicate no difference in the availability of iron from the two sites (5, 6). Thus far, most of the nonbiological probes indicate that the two iron-binding sites in transferrins are nonequivalent (2).

One direct approach for proving the equivalence or nonequivalence of sites has been to release the individual iron-binding domains from the transferrins by means of chemical and proteolytic procedures, and to investigate them separately. The first iron-binding fragment was isolated from apo-ovotransferrin by cyanogen bromide cleavage, and represents the NH₂-terminal domain of the molecule. The iron complex of the fragment, however, showed a significant change in the visible absorption spectrum, indicating a structural alteration in the iron-binding site (7, 8).

The proteolytic approach was initially introduced by Azari and Feeney (9), who showed that the saturated iron complexes of OT² and serum transferrin are significantly more resistant to proteolysis by chymotrypsin and trypsin than OT. Similar observations were reported by Williams (10).

Proteolysis of partially saturated iron complex has been found to liberate iron-binding fragments; however, the type of fragments liberated depends on the source of transferrin, extent of its saturation with iron, and the nature of the protease.

For example, tryptic and chymotryptic digestion of partially iron-saturated human transferrin and OT have been shown to cause liberation of NF (10, 11), whereas tryptic cleavage of pig and bovine transferrins, either as iron-saturated or apo-proteins, produces NF and CF (12, 13). NF and CF have also been obtained from human lactoferrin by pepsin, trypsin, and chymotrypsin action (14). The action of subtilisin on Fe₂OT releases only CF (15), whereas only NF is released from a thermolysin digest of iron-saturated human transferrin (16).

For the investigation of the iron-transferrin activity of NF and CF in a homologous chicken embryo red cell system (17), it was necessary to produce sufficient quantities of pure fragments possessing the structural integrity and iron-binding activity of the native molecule. In our experience, the procedures described by Williams (10, 15) systematically gave low yields of NF, and in the case of CF, the subtilisin digest gave multichain iron-binding fragments which were contaminated with residual active subtilisin, even in the presence of PMSF.

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1 In this report, iron-binding fragments NF and CF denote the iron binding domains from the NH₂- and CO₂H-terminal halves of ovotransferrin.

2 The abbreviations used are: OT, apo-ovotransferrin; Fe₂OT, iron-saturated OT; NF and CF, apo-NH₂-terminal and apo-CO₂H-terminal fragments, respectively; FeNF and FeCF, fragment containing one atom of ferric iron per molecule; TN, NH₂-terminal fragment produced by trypsin hydrolysis of 30% saturated iron-OT complex; Sub I, II, III, fragments produced from subtilic digestion of Fe₂OT; CNBr, cyanogen bromide; DPCC, diphenylcarbomyl chloride; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Fig. 1. Chromatographic separation of FeNF and FeCF. A, fractionation pattern of the subtilitic digest of FeOT on a Sephacryl S-200 superfine matrix (2.5 x 200 cm). Elution buffer was 50 mM ammonium acetate, pH 8.0. Chromatography was run at room temperature and fractions of 6 ml were collected at a flow rate of 30 ml/h at room temperature. B, fractionation pattern of the iron-binding fragments (from peak II. A) on a DE32 cellulose matrix (1.5 x 25 cm) with a linear gradient of 10 to 100 mM ammonium acetate, pH 8. Fractions of 5 ml were collected at a flow rate of 20 ml/h at room temperature. Horizontal bars indicate fractions pooled.

To avoid contamination of fragments by subtilisin and to increase the yield, we have prepared and employed Sepharose-immobilized subtilisin Novo. This modification was not only successful in eliminating contamination but also produced a good yield of NF and CF in a single operation. In this report, we present the subtilic procedure for the production of NF and CF and their purification. The physical, chemical, and immunochemical properties of the fragments are also presented, some of which have not been reported previously. The iron-transferring activity of the fragments, in the chick embryo red cell system, is presented elsewhere (17).

EXPERIMENTAL PROCEDURES3

RESULTS

Purification of NF and CF from Subtilitic Digests of FeOT—The elution profile of a subtilitic digest of FeOT on a Sephacryl S-200 superfine column is shown in Fig. 1A. The first peak represents intact FeOT, the second peak iron-binding fragments of $M_r \approx 40,000$, and the third peak fragments with smaller molecular weights. The $A_{280}/A_{465}$ ratios of peaks I and II are 24.5 and 26.4, respectively, indicating that both fractions were essentially saturated with specifically bound iron. Peak III did not show absorbance at 465 nm and was not studied further. The yields were: peak I, 35%; peak II, 40%; peak III, 12%.

DE-cellulose chromatography of pooled fractions from peak II produced three major peaks: Sub I, Sub II, and Sub III, as shown in Fig. 1B. The protein distribution of eluted peaks was: Sub I, 35%; Sub II, 28%; Sub III, 37%. Fractions under each of these peaks were collected and further purified by rechromatography on the same column. The purified fragments were essentially homogeneous as judged by polyacrylamide gel electrophoresis in the discontinuous Tris-glycinate system (Fig. 2A). SDS-gel electrophoresis patterns of FeOT, Sub I, Sub II, and Sub III, before and after CNBr cleavage, are shown in Fig. 2B. They all show a single band in SDS gel before CNBr cleavage. The molecular weight of FeOT is $\sim 78,000$ and the molecular weight of each of the three iron-binding fragments $\sim 39,000$. Reduction and carboxymethylation of FeOT and the iron-binding fragments produced no additional bands (result not shown), indicating that they all have single-chain structures.

As seen upon CNBr cleavage, Sub II yields a major fragment of $M_r = 35,000$, whereas Sub II and Sub III produce smaller fragments ranging in $M_r = 13,000$ to $23,000$. While Sub II and Sub III have almost identical CNBr cleavage patterns, they share no common CNBr fragment with Sub I. Furthermore, the CNBr cleavage pattern of FeOT is approximately equal to the sum of Sub I and Sub II or Sub I and Sub III. Based on these results and the reported CNBr cleavage pattern of OT (7), it is suggested that Sub I is derived from the NH$_2$-terminal domain of OT and is very similar to NF produced by tryptic action, whereas Sub II and Sub III are derived from the CO$_2$H-terminal domain of OT and represent CF.

Purification of NF from Tryptic Digest of Iron-OT—The Sephacryl and DE-cellulose procedures, described for a subtilic digest, were also employed for the purification of NF (also designated as TN) from the tryptic digest of FeOT. Sephacryl elution also produces three peaks (similar to Fig. 1A). The second peak contains the NF. The NF fraction was further chromatographed on a DE-cellulose column with 10 to 100 mM, pH 8, ammonium acetate gradient. The major peak was rechromatographed under the same conditions and produced a homogeneous NF. The purified NF has an $A_{280}/A_{465}$ ratio of $\sim 25$ which is close to the value for the native FeOT.

NF shows a single band in the discontinuous Tris-glycinate gel system. SDS-gel electrophoresis of the reduced and carboxymethylated NF also shows a single band of $M_r = 39,000$. This indicates that NF has a single-chain structure, representing approximately half the FeOT molecule. Furthermore, CNBr cleavage of NF yields a major fragment with a $M_r = 35,000$, which indicates that NF is derived from the NH$_2$-terminal domain of OT (7, 8).

Amino Acid Composition, NH$_2$-terminal Residues, and Carbohydrate Content of Iron-binding Fragments—The amino acid compositions of the iron-binding fragments are shown in Table I. The composition of OT is also shown for comparison. The amino acid compositions of the two NFs (produced by tryptic or subtilic digestion) are almost identical to each other and the same is true for the two CFs. In general, all fragments show close similarity in composition except for alanine, histidine, and methionine.

The NH$_2$-terminal amino acid residues of OT and the iron-binding fragments are also shown in Table I. While the two NFs have the same NH$_2$-terminal residue as OT, i.e. alanine, the NH$_2$-terminal residues of Sub II and Sub III are threonine and aspartate, respectively. It should be pointed out that in
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3, lane their CNBr-cleaved products. Plates for NFs (TN and Sub I), whereas for Sub I1 and Sub I11 was the only dansylamino acid detected on the polyamide. The NH2-terminal amino acid determinations, dansylalanine is seen in Fig. 4, when wells 2 and 6 contained Sub I and TN, respectively. These results indicate immunochemical identity between Sub I1 and Sub I11, besides threonine and aspartate, several unidentified fainter fluorescent spots were also observed.

Results of carbohydrate analysis of the iron-binding fragments are also included in Table I. It is seen that almost all the carbohydrate of OT is present in CF.

Immunodiffusion Patterns of FeNF, FeCF, and Fe2OT—Immunodiffusion patterns of FeNF, FeCF, and Fe2OT against anti-Fe2OT are shown in Fig. 3. Continuous precipitin bands are seen in Fig. 3A when wells 2 and 3 (or 5 and 6) contained Sub II and Sub III, respectively. Similarly, in Fig. 3B continuous precipitin bands are seen when wells 2 and 3 (or 5 and 6) contained Sub I and TN, respectively. These results indicate immunochemical identity between Sub II and Sub III and Sub I and TN. As is also seen in Fig. 3, A and B, precipitin bands between Fe2OT (wells 1 and 2) and any of the fragments produced a spur which indicates a partial immunochemical identity of fragments with Fe2OT.

Fig. 3C shows a continuous precipitin band between a mixture of NF and CF (wells 2, 3, 5, and 6) and Fe2OT (wells 1 and 4). These results indicate that although NF and CF are immunochemically different and contain only a portion of the antigenic specificity of the OT molecule, their mixture provides a full antigenic specificity of the intact OT molecule.

Rate of Iron Release from the Fragments—Iron release from FeNF, FeCF, and Fe2OT was induced by the addition of citric acid and followed by measuring the decrease in A650. Since TN and Sub I are essentially identical to each other, as are Sub II and Sub III, results from only one of each group are shown. Fig. 4 shows that the release of iron from FeNF is faster than from FeCF, and the rate of iron release from Fe2OT very similar to FeNF. The theoretical sum of the curves of the two fragments is substantially different from that of the intact protein, but very similar to the experimental curve of a 1:1 mixture of FeNF and FeCF.

Iron-binding Capacity of NF and CF and Their Iron Complexes—As shown in Fig. 5 A, FeNF and FeCF are highly resistant to trypsin hydrolysis, as compared to the apo-fragments. In 20 min, the apo-fragments show a 75 to 80% loss in chromogenic activity, whereas their iron complexes show very little loss in chromogenic activity for the same time period. A further exposure of FeNF and FeCF to trypsin for 2 h shows only 4 and 3% loss in chromogenic activity, respectively.

Fig. 5B shows the rate of loss of chromogenic activity of the fragments and their iron complexes in the presence of subtilisin. Once again, the iron complexes are more resistant to subtilisin hydrolysis than the apo-fragments. However, subtilisin destroys the chromogenic activity of both FeNF and FeCF at a faster rate than trypsin. Furthermore, FeNF is hydrolyzed significantly faster than FeCF.

Spectroscopic Studies—The visible absorption spectra (not shown) of Fe2OT, FeNF, and FeCF show very similar charge transfer bands with a maximum at 465 nm and essentially identical absorptivity.

Fig. 6A shows the CD spectra of Fe2OT, FeNF, and FeCF in the visible region. A strong negative CD band at 450 nm is observed for all three proteins. The presence of the CD band associated with the charge-transfer absorption band indicates that iron-binding is asymmetric. Compared to the corresponding absorption band, however, the CD band has shifted 15 nm to the blue. Two intense positive CD bands are also seen at 305 and 325 nm for all three iron proteins, with FeNF showing a stronger band at 305 and weaker band at 325, as compared to FeCF. The CD spectra in the 250 to 300 nm region are shown in Fig. 6B. The spectra of Fe2OT and the two iron-binding fragments are very similar to one another. The negative CD bands at 286 and 277 nm, which could be due to protonated tyrosine and probably also tryptophan residues, are observed in all three spectra. A positive CD at 295 nm, which is very unusual, is also observed for Fe2OT and the iron-binding fragments. Fig. 6C shows the CD spectra in the 195 to 245 nm region. For FeCF, the negative band at 218 nm is diminished and becomes a shoulder to the main band at 210 nm.

The percentage of various structures in Fe2OT and its iron binding fragments are shown in Table II. Results from the two methods indicate that the percentage of β-sheet structure in Fe2OT and in the two iron-binding fragments is about the same but there is a substantial difference in their α-helix.
content. FeCF shows a two-fold higher α-helical content than FeNF.

The EPR spectra of FeNF and FeCF are shown in Fig. 7A. The spectrum of FeCF shows a main line around \( g = 4.2 \) and a strong shoulder on the low-field side of the main line. A broad signal around \( g = 8.8 \) is also observed. The spectra of FeNF also show a sharp signal around \( g = 4.2 \). However, its intensity is much lower than that of FeCF. The intensity of the strong shoulder and the broad line around \( g = 8.8 \) of FeNF, however, is higher than those of FeCF. Fig. 7B shows the EPR spectra of FeOT and a 1:1 mixture of FeCF and FeNF. The two spectra are closely superimposable.

**DISCUSSION**

Immobile subtilisin seems to be the most efficient system to date for the production of native iron-binding domains from FeOT. The yield of fragments is ~40%, which is significantly higher than by the tryptic procedure of Williams (10, 15). Furthermore, FeNF and FeCF are obtained simultaneously by a single enzymatic digestion. Subtilisin digestion yields less NF (13%) than CF (27%), apparently due to the higher susceptibility of FeNF to further subtilisin digestion than FeCF (Fig. 5B).

Since mechanical force such as stirring and shaking may destroy the solid matrix of the immobilized enzyme and release the attached enzyme into the solution, the digestion was carried out in a column. Usually a small volume of concentrated FeOT solution (10%) was applied to the column. However, the procedure could be adapted to the continuous application of a larger volume of FeOT. The coupling and hydrolysis were performed at least 4 times, and in each case, the coupled enzyme had a similar activity toward \( p \)-nitrophenylacetate (30 to 40% of the soluble enzyme) and produced similar hydrolytic fragments from FeOT.

The accessibility of the substrate to the matrix-bound enzyme was improved by maintaining a continuous flow of substrate through the column. This procedure eliminated the necessity for the complete removal or inhibition of the enzyme after digestion. The finding that insignificant internal cleavage had occurred in the iron-binding fragments suggests that the reaction catalyzed by the solid state enzyme is more restrictive than in solution. The restrictive action of immobilized enzyme may be due to its decreased activity, as well as a change in its specificity.

It has been shown previously that a 35,000-dalton CNBr fragment is derived from the NH₂-terminal domain of OT (7, 8). Since both NFs (TN, prepared by tryptic procedure of Williams (10), and Sub I, isolated in the present study) yielded a 35,000-dalton fragment upon CNBr treatment, it was concluded that they originated from the NH₂-terminal domain of the OT molecule. On the basis of the finding that Sub II and Sub III are complementary to the NFs (TN and Sub I) in immunological properties and CNBr cleavage patterns, it was concluded that they are derived from the COOH-terminal domain of OT.

The production of NF by the tryptic digestion of partly iron-saturated OT is in general similar to the procedure of Williams (10); however, the \( M_r = 39,000 \) found for NF in our investigation is ~4000 higher than reported. Furthermore, the yield of NF is only 4%, and the final digest contains 20% FeOT. One explanation for the difference may be the use of iron citrate in our investigation instead of iron-nitrilotriacetate (used by Williams) for the preparation of partly saturated FeOT complex. It has been reported by Donovan et al. (35) that iron nitrilotriacetate complex promotes indiscriminate and anticooperative binding of iron to OT and in its absence the binding becomes positively cooperative.

A CF has also been isolated by limited tryptic digestion of an OT. Fe complex (15). According to this procedure, at pH 5, FeOT releases iron preferentially from the NH₂-terminal site to give OTFe, which upon digestion by trypsin produces CF. Attempts to produce CF by this procedure were unsuccessful. Instead of CF, we obtained NF (similar to tryptic digestion). While these results are in contrast to the acid-induced iron release data for FeNF and FeCF, which indicate a higher stability for FeCF, they are in accord with the iron release data for intact FeOT, which indicate a significant increase in the rate of iron release from the COOH-terminal domain. (Fig. 4). The finding that the rate of acid-induced iron release from FeOT is much faster than from a 1:1 mixture of FeNF and FeCF suggests that the release of iron from the two sites of intact FeOT is cooperative rather than independent.

NF and CF differ in amino acid and carbohydrate composition, CNBr cleavage pattern, rate of acid-induced iron release, and immunological properties. Despite the compositional and stability differences between the NF and CF, their iron-binding environments are very similar to each other as evidenced by the similarity in their absorption and the CD spectra in the visible region. The negative CD band of the charge-transfer transition is not located at 465 nm, as the corresponding absorption band, but is blue-shifted to 450 nm, which may indicate that the charge-transfer band consists of multiple components. Besides the negative CD band at 450 nm, two positive CD bands are also observed at 305 and 325 nm. Since they are not observed in the apoproteins, they are also induced by iron binding. It has been suggested (36) that they are due to at least one of the disulfide bonds in OT which undergoes changes in dihedral angle upon iron-binding. The unequivocal assignment of CD bands in the 250 to 300-nm region is difficult. On the basis of studies of amino acids and proteins (37), the 295-nm band is most likely due to tryptophanyl residues. The 286 and 277-nm bands may arise from protonated tyrosyl and perhaps tryptophanyl residues. The 253 nm band is assigned tentatively to disulfide bonds. The positive ellipticity of the 295 nm band is probably due to the superposition of the intense charge-transfer CD band around 305 nm to this region of the spectra.

The CD spectrum in the 190 to 250 nm region depends primarily on the secondary structure of the protein. The CD spectrum of FeOT obtained in this region is very similar to that observed by Tan (36), except that a more profound band around 218 nm was observed in the present study which is unusual and it may indicate the presence of high content of \( \beta \) structure. The α-helix content of FeOT calculated by the method of Chen et al. (23) is 14%, which is very close to the values of 15 and 16% reported by Tan (36) and Nagy and Lehrer (38), respectively.

The spectroscopic and immunochemical data strongly suggest that the proteolytic cleavage of FeOT into separate domains has not induced significant changes in the conformation and iron-binding activity of the domains.

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**REFERENCES**

Iron-binding Fragments of Ovotransferrin

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S. M. 5-200 Superfine and Sepharose 4B (50-100 mesh) were obtained from Pharmacia, DSS Cellulose anion exchanger (Whatman DE52 Cellulose) was a product of Whatman Chemical Co. Ovotransferrin from hen egg white in 0.1 M sodium acetate, pH 5.6, DSS-DE52-treated tryptophan and FNW were purchased from Sigma Chemical Co. Nucleopore filters with 0.20 um pores were obtained from Rainin Instrument Co. New York.

Preparation of Protein

The purpose of the present study was to obtain iron-binding fragments of ovotransferrin. Iron-binding fragments were obtained from a culture of the Bacillus subtilis mutant 148 I-7. I-7 refers to the strain which produces an iron-binding protein with a molecular weight of about 28,000. The iron-binding protein was purified by DEAE-cellulose column chromatography. The iron-binding protein was then subjected to electrophoresis on a SDS-polyacrylamide gel. The iron-binding fragments were then obtained by running the iron-binding protein on a cellulose acetate membrane.

Iron-binding Fragments of Ovotransferrin

The iron-binding fragments were obtained from a culture of the Bacillus subtilis mutant 148 I-7. The iron-binding fragments were obtained by subjecting the iron-binding protein to electrophoresis on a SDS-polyacrylamide gel. The iron-binding protein was then subjected to electrophoresis on a cellulose acetate membrane. The iron-binding fragments were obtained by running the iron-binding protein on a cellulose acetate membrane.

Iron-binding Fragments of Ovotransferrin

The iron-binding fragments were obtained from a culture of the Bacillus subtilis mutant 148 I-7. The iron-binding fragments were obtained by subjecting the iron-binding protein to electrophoresis on a SDS-polyacrylamide gel. The iron-binding protein was then subjected to electrophoresis on a cellulose acetate membrane. The iron-binding fragments were obtained by running the iron-binding protein on a cellulose acetate membrane.

Iron-binding Fragments of Ovotransferrin

The iron-binding fragments were obtained from a culture of the Bacillus subtilis mutant 148 I-7. The iron-binding fragments were obtained by subjecting the iron-binding protein to electrophoresis on a SDS-polyacrylamide gel. The iron-binding protein was then subjected to electrophoresis on a cellulose acetate membrane. The iron-binding fragments were obtained by running the iron-binding protein on a cellulose acetate membrane.

Iron-binding Fragments of Ovotransferrin

The iron-binding fragments were obtained from a culture of the Bacillus subtilis mutant 148 I-7. The iron-binding fragments were obtained by subjecting the iron-binding protein to electrophoresis on a SDS-polyacrylamide gel. The iron-binding protein was then subjected to electrophoresis on a cellulose acetate membrane. The iron-binding fragments were obtained by running the iron-binding protein on a cellulose acetate membrane.
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Fig. 5 (A) Purity of Fe-binding and (B) inactive equivalents of the ovotransferrin fragments of Ff, Ff(1-45), Ff(1-53), Ff(1-57), or Ff(1-57). (See Experimental Procedures.)

Table 1: The percentages for different conformations were computed from the far UV CD spectra for each protein (Fig. 5C).

Fig. 6 (A) CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-). (B) CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-). The concentrations of the samples were about 0.1 mg/ml in 0.05 M sodium phosphate, pH 7.0 buffer. The cell pathlength was 1 cm and ellipticity was recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm.

Fig. 7 The CD spectra of frozen solutions of Ff(1-45), Ff(1-53), and Ff(1-57) were recorded at a temperature close to 273 K with a variable range spectrometer operating near 0.02 cm. The millimeter path was 0.05 cm. The CD spectra of Ff(1-45) (-), Ff(1-53) (-), and Ff(1-57) (-) were recorded at 2.5 cm.